*Environmental Health Perspectives*
*Vol. 93, 3–10, 1991*

# Alterations in Epidermal Biochemistry As a Consequence of Stage-Specific Genetic Changes in Skin Carcinogenesis

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The induction of cancer on mouse skin by initiation-promotion protocols occurs through stages in which a benign squamous papilloma is an obligate precursor of squamous cell carcinoma. Activation of the Ha-*ras* gene is sufficient to produce the papilloma phenotype, while additional genetic changes are required for malignant conversion. The introduction of Ha-*ras* into normal keratinocytes suppresses the expression of differentiation markers, keratin K1 and K10, and loricrin (a cornified envelope precursor) and, to a lesser extent, filaggrin, at the level of transcription. However, cells initiated by Ha-*ras* express a nonepidermal keratin, K8. The transcription of K8 in these cells is sensitive to the level of medium $Ca^{2+}$, being abundant in 0.5 mM $Ca^{2+}$ and not detected in 0.05 mM $Ca^{2+}$. Epidermal differentiation is regulated by signalling, which involves changes in phosphatidylinositol turnover and intracellular $Ca^{2+}$. Cells initiated by Ha-*ras* do not differ from normal keratinocytes in their intracellular $Ca^{2+}$ response patterns, at least in response to changes in extracellular $Ca^{2+}$ and serum factors. However, c-Ha-*ra* keratinocytes have a high basal level of phosphatidylinositol (PI) turnover, which is additive with several other inducers of this pathway, including $Ca^{2+}$ and aluminum fluoride. Additional studies suggest that high turnover of the PI pathway is incompatible with differentiation-specific gene expression in keratinocytes. We suggest this negative relationship is mediated through elevated diacylglycerol production and chronic down-modulation of protein kinase C. Protein kinase C is known to be essential for expression of differentiation-related genes in keratinocytes.

Several approaches have been taken to evaluate genes involved in malignant conversion. Stable papilloma cell lines, which express a codon 61 A to T transversion mutation in the Ha-*ras* gene, were used as recipients of exogenous cloned oncogenes. The *E1A* and *myc* genes did not alter the tumor phenotype when transfected cells were tested *in vivo*. In contrast, two transforming constructs of v-*fos* caused malignant conversion, while c-*fos* was ineffective in this regard. He-*ras* and v-*fos* were also introduced into normal keratinocytes using defective retroviruses and the recipient cells tested *in vivo* for tissue phenotype. Co-infected cells produced carcinomas, v-Ha-*ras* alone produced papillomas, and v-*fos* alone produced normal skin. The capacity of transforming *fos* constructs to cause malignant progression in benign cells with a Ha-*ras* mutation suggests an indirect mechanism through activation of transcription of cellular genes. Among the *fos*-regulated gene family are secreted proteases, and several of these enzymes are elevated in tumors converted by the combined action of *fos* and *ras* oncogenes. These results suggest the possibility that activation of the protease cascade could occur early in malignant progression. The disruptive consequences of active protease secretion on extracellular regulatory processes could account for the disordered expression of keratinocyte-specific genes in carcinomas.

## Introduction

The induction of tumors by the application of chemicals to the skin of mice has revealed fundamental concepts regarding the biology of chemical carcinogenesis. Cancer induction in this tissue requires at least three distinct stages: initiation, promotion, and malignant

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conversion. Initiation occurs rapidly, is irreversible, and is commonly caused by mutagens. Initiation persists throughout the lifetime of the animal but may not result in tumors in the absence of further treatment.

Recently, a point mutation in the c-Ha-*ras* gene has been strongly linked to the initiated phenotype in skin (*1–3*). Promotion results from the application of promoting agents that are not mutagenic in most cases. Promotion must occur after initiation and requires repeated exposures to effective agents with an obligate frequency. The effects of individual promoting exposures are reversible. Promoters alter tissue homeostasis and provide an environment for the selective outgrowth of initiated cells.

The clinical consequence of initiation and promotion is the production of multiple benign tumors, squamous papillomas, each representing the clonal expansion of single initiated cells. Papillomas may persist or regress and infrequently undergo malignant conversion to squamous carcinomas. Since the low frequency of spontaneous malignant conversion can be enhanced by exposure of papilloma-bearing mice to mutagens (*4,5*), the conversion event is likely to represent additional genetic changes in the initiated cell population. Because multiple genetic changes in the same cell are required to produce a cancer cell, the epigenetic process of tumor promotion is the rate-limiting step in cancer development *in vivo*. By increasing the clone size of the initiated population expressing a relevant mutation, promoters enhance the probability that a second genetic change will occur in that population.

# Tumor Phenotype *In Vivo*

## Markers Expressed by Tumors

Considerable data have accumulated on the phenotypic alterations produced in skin cells during the multistage development of squamous cancer (Table 1). In normal skin, two keratins, K5 (Mr 60,000) and K14 (Mr 55,000), are transcribed largely in basal cells, although the proteins persist in the upper layers (*6*). The commitment to differentiate is associated with the transcription of two suprabasal keratins, K1 (Mr 67,000) and K10 (Mr 59,000), in the first spinous layer (*6*). Transcripts for K1 and K10 diminish as cells migrate into the granular cell layer where K1 and K10 proteins are polymerized into tonofilament bundles. In this layer, new proteins are expressed including filaggrin, an Mr 27,000, interfilamentous matrix protein, and loricrin, a major component of the cornified envelope (*7,8*).

**Table 1. Expression of differentiation markers in tumors.**

| Marker protein | Normal *in vivo* expression | Expression in tumors | |
|---|---|---|---|
| | | Papillomas | Carcinomas |
| K5 | Epidermal basal layer | ++++ | ++++ |
| K14 | | ++++ | ++++ |
| K1 | Epidermal spinous layer | ++ | − |
| K10 | | ++ | − |
| Filaggrin | Epidermal granular layer | ++ | − |
| Loricrin | | ++ | − |
| K6 | Hyperproliferative epidermis | +++ | ++++ |
| K13 | Internal stratified epithelium | − | +++ |

In benign tumors produced by chemical initiation, the distribution of K14 protein is similar to that in normal skin, while the abundance of K1 and K10 is reduced although the tissue distribution remains suprabasal (*6*). Transcription of K14 is aberrant in benign tumors since transcripts persist throughout many of the suprabasal cell layers (*6*). Transcripts for K1 and K10 are low in the first suprabasal layer of papillomas, unlike in normal skin where they are abundant. Some papilloma cells that express K1 or K10 are capable of proceeding through S-phase as indicated by the incorporation of BrdU into nuclei of K1-positive cells after pulse labeling. Thus, papilloma cells display an altered response to signals that in normal cells trigger the early transcription of differentiation-specific genes and the inhibition of proliferation-specific functions. The pattern of expression for loricrin in benign tumors is similar to that for normal skin, although loricrin protein levels are reduced. Benign tumors commonly display many filaggrin-positive cell layers (*9*). Thus, expression of the late differentiation-related genes is less disturbed in benign neoplasms than that of the early differentiation markers.

Keratin expression in carcinomas is highly disturbed (*6*). K14 protein and transcripts are diffusely expressed in carcinomas, while protein and transcripts for K1 and K10 are essentially absent. Both loricrin and filaggrin proteins are greatly diminished in carcinomas, and transcripts are in low abundance. The near absence of detectable transcripts and proteins for suprabasal keratins, loricrin, and filaggrin provides a marker for malignant conversion in the mouse skin carcinogenesis model. By immunofluorescence analysis, foci negative for expression of these markers can be noted prior to detection of a definitive change in cellular phenotype by light microscopy, suggesting that these are early events that characterize malignant conversion (*6*). In addition, carcinomas express keratins, such as K13 and K19, which are never expressed in normal skin but are characteristic of internal epithelia (*10*). By contrast, the proliferation rate (as measured by labeling index) in carcinomas is similar to that of papillomas (*11*), suggesting that the changes in differentiation-related gene expression are central to the conversion phenotype.

# Characteristics of the Initiated Phenotype

Considerable insight regarding the regulation of normal skin growth and differentiation has evolved from studies of keratinocytes in cell culture (*12*). These studies have revealed that extracellular $Ca^{2+}$ is a major determinant of the differentiation state of epidermal cells. Under conditions of $Ca^{2+}$ reduced to 5% of the

level in serum (0.05 mM), keratinocytes have a basal cell phenotype. At $Ca^{2+}$ of 0.12 mM, the suprabasal markers are induced in a process regulated transcriptionally (*13*). The expression of suprabasal markers is coordinated in a temporal sequence similar to that seen in skin *in vivo* (*13*). When extracellular $Ca^{2+}$ is > 0.3 mM, cultured keratinocytes differentiate, but expression of the suprabasal markers is much reduced. Differentiating cells stop proliferating, form cornified envelopes, and slough from the culture dish probably due to activation of the envelope-crosslinking enzyme, epidermal transglutaminase (*14*). Epidermal cells from initiated skin and benign or malignant skin tumors, as well as cells exposed to chemical carcinogens *in vitro*, are resistant to the induction of differentiation by $Ca^{2+}$ *in vitro* (*15*). This has formed the basis for a method to select for cells with the neoplastic phenotype (*16*) and is consistent with a defect in the response to differentiation signals as a fundamental change in neoplastic transformation of skin cells (*17*).

## Keratinocyte Phenotype Produced by Activation of the Ha-*ras* Gene

We have used the Ha-*ras* gene to analyze the phenotype of initiated keratinocytes *in vitro*. When introduced into normal cultured mouse basal keratinocytes (0.05 mM $Ca^{2+}$) by a defective retroviral vector, v-Ha-*ras* increases the proliferation rate of the cultured population by 5-fold (*18*). Furthermore, the infected cells are resistant to terminal differentiation in 1 mM $Ca^{2+}$ medium, although the proliferation rate is markedly reduced at that concentration of $Ca^{2+}$ (*19*). Transfer of v-Ha-*ras* keratinocytes from culture to a prepared skin graft bed produces papillomas on recipient mice (*20*), consistent with the complete initiating activity of this single genetic change.

Additional studies have now been performed to characterize the v-Ha-*ras* phenotype *in vitro* (Table 2). When infected basal cells (0.05 mM $Ca^{2+}$) are switched to 0.12 mM $Ca^{2+}$, the expression of both protein and mRNA for the suprabasal keratin markers K1 and K10 and the granular cell marker loricrin is inhibited. Thus, v-Ha-*ras* impairs the pathways involved in response to a differentiation signal. Expression of the granular cell-specific filaggrin protein and mRNA is less consistently affected by the exogenous v-Ha-*ras* oncogene at the permissive $Ca^{2+}$, suggesting that filaggrin might have distinct control mechanisms in addition to its common $Ca^{2+}$ regulation with the other markers. Infection of cells with an identical retroviral vector containing only a *neo*$^r$ gene did not alter the differentiation response to 0.12 mM $Ca^{2+}$.

**Table 2. Phenotypic and metabolic characteristics of keratinocytes initiated by a v-Ha-*ras* oncogene.**

| Marker | Changes produced by v-Ha-*ras* |
|---|---|
| *In vivo* | |
| Skin graft | Papilloma |
| Suprabasal keratins | Expression reduced |
| *In vitro* | |
| Labeling index | Increased 5-fold |
| Basal cell keratins | Expressed |
| Suprabasal keratins | Absent |
| Loricrin | Reduced |
| Filaggrin | Variable |
| Keratins 8 and 18 | Expressed (not detected in normal keratinocytes) |
| Intracellular $Ca^{2+}$ response | Normal |
| Phosphatidylinositol turnover | Increased 2-fold |
| Diacylglycerol content | Increased 6-fold |

Cytoskeletal extracts of v-Ha-*ras* keratinocytes contain a novel 58 kD protein band that is also sensitive to $Ca^{2+}$. Western blotting of relevant cell extracts indicates that the 58 kD protein is keratin 8 (K8) because it reacts with two monoclonal antisera (35 βH 11 and Troma 1) specific for that keratin marker (*21,22*). Previously, keratin 8 was shown to be expressed in SV-40 immortalized human keratinocytes (*23*) and transiently in developing human hair follicles (*24*). K8 transcripts are present in epidermal cells transduced with v-Ha-*ras* and cultured in 0.5 mM $Ca^{2+}$ medium but not in normal keratinocytes. These results are consistent with a $Ca^{2+}$-dependent change in the program of epidermal gene expression in these initiated cells.

## Intracellular Signaling Pathways in v-Ha-*ras* Keratinocytes

The expression of differentiation markers in cultured keratinocytes is tightly linked to specific changes in intracellular $Ca^{2+}$ ($Ca_i$) and phosphatidylinositol (PI) metabolism (*25–27*). However, the immediate $Ca_i$ response to a change in extracellular $Ca^{2+}$ is identical in control and v-Ha-*ras* keratinocytes when measured by digital image analysis of changes in fluorescence of the $Ca^{2+}$-sensitive dye, Fura 2 (*28*). Sustained $Ca_i$ responses over a 24-hr measurement period are also nearly identical for v-Ha-*ras* and control keratinocytes. A pharmacologically induced increase in $Ca^i$ caused by exposure to 6.5 μM ionomycin did not restore expression of differentiation markers in v-Ha-*ras* cells but did cause the cells to cornify and slough from the culture dish. This suggests that the lack of marker expression in v-Ha-*ras* cells is not due to a resetting of the $Ca_i$ requirements for gene activation to a higher level. Together these results indicate that a direct alteration in $Ca_i$ response does not account for the influence of v-Ha-*ras* on keratinocyte gene expression.

In other cell types, the Ha-*ras* oncogene can influence phosphatidylinositol metabolism, probably by virtue of its G protein character (*29–31*). Whether this is specific to *ras* oncogenes and germane to their transforming activity is in dispute. However, G proteins are likely to be essential in the activation of the phospholipase C, which regulates phosphatidylinositol metabolism. The relevance of PI metabolism to the differentiation of keratinocytes (*25–27*) suggests that alterations in this pathway via Ha-*ras* activation could be important in producing the initiated phenotype.

To explore the influence of v-Ha-*ras* on keratinocyte

PI metabolism, equilibrium labeling of cultured cells with $^3$H-inositol and HPLC analysis of labeled cell extracts were performed. Introduction of v-Ha-*ras* into normal keratinocytes increases basal levels of inositol phosphates over controls in 0.05 mM $Ca^{2+}$, indicating a greater steady-state rate of PI metabolism. Turnover is increased even further when the cells are switched to 0.12 mM or 1.4 mM $Ca^{2+}$. The incremental increase above the already elevated basal level of PI turnover by an increase in extracellular $Ca^{2+}$ suggests that v-Ha-*ras* and $Ca^{2+}$ are not stimulating PI metabolism via the same effector, although partial effects for each cannot be ruled out. Aluminum fluoride (Al/F) also stimulates basal PI metabolism in normal cells even more potently than $Ca^{2+}$ (*32*). The Al/F results suggest that G protein activation is important in PI turnover in keratinocytes (*33*). Al/F also inhibits the expression of suprabasal markers of differentiation in keratinocytes. These results suggest that excessively elevated levels of PI metabolites may be inhibitory for expression of differentiation markers in skin cells.

The influence of v-Ha-*ras* and certain pharmacological agents on the expression of keratinocyte-specific differentiation markers may provide a clue to the pathways influenced by the activation of Ha-*ras* during initiation of carcinogenesis. Conditions that are nonpermissive for the expression of specific epidermal markers are associated with high activity of the PI cycle or high intracellular $Ca^{2+}$. Additionally, conditions which downmodulate protein kinase C, such as pretreatment of keratinocytes with phorbol esters or bryostatin abrogate the keratinocyte response to an appropriate $Ca^{2+}$ signal for marker expression *in vitro* or to the physiological signals *in vivo* (*34,35*).

Previously, we have shown that initiated keratinocyte cell lines from a variety of sources are resistant to the induction of terminal differentiation by phorbol esters and are defective in expression of differentiation markers (*36,37*). Some of these lines, as well as v-Ha-*ras* cells, are stimulated to proliferate by phorbol esters, but the magnitude is usually small (*19,36*). Furthermore, a limited number of initiated cells so far examined have phorbol ester binding profiles that differ from normal cells (*38*). Consistent with the above results are reports in other systems where the introduction of a *ras* oncogene stimulates PI turnover (*29*), elevates diacylglycerol levels (*30,31*), and causes a decrease in protein kinase C (*31*). Microinjection of protein kinase C can restore such cells to a control response pattern (*39*). Together these findings suggest that the initiated phenotype in keratinocytes is associated with the inactivation (partial or total), subcellular redistribution, or changed isozyme expression of protein kinase C. Studies to test this hypothesis are currently in progress.

# Malignant Conversion

## Genetic Basis for Malignant Conversion

Several lines of evidence suggest that a single genetic change is sufficient to cause malignant progression in skin carcinogenesis (Table 3). Papilloma-derived cell lines, which do not express a detectable oncogene, advanced to anaplastic carcinomas when transfected with the human EJ bladder carcinoma Ha-*ras* oncogene (*40*). Furthermore, the c-Ha-*ras* mutation, commonly heterozygous in chemically induced papillomas, is frequently homozygous and amplified in chemically induced squamous carcinomas (*41*), suggesting that Ha-*ras* oncogene dosage is important in determining tumor phenotype. These conclusions are consistent with *in vitro* studies indicating that two different oncogenes may act in concert to achieve malignant transformation of primary rat embryo cells (*42*) and that the required combinations are specific. The introduction of certain viral oncogenes into Syrian hamster cells previously immortalized by carcinogens (*43*) causes malignant progression, suggesting that chemically induced mutations can cooperate with cloned oncogenes. The cooperative action of pairs of oncogenes has suggested the existence of complementation groups in which some genes alter cells to a phenotype that is not tumorigenic (e.g., adenovirus E1A and polyoma large T-antigen), while others impart a neoplastic phenotype (e.g., the *ras* oncogene family and polyoma middle T) (*44*). These groupings have evolved from studies of mesenchymal cells.

## Analysis of Malignant Conversion by Oncogene Transfection into Neoplastic Keratinocytes

The analysis of malignant conversion *in vivo* is particularly problematic because the time between a conversion event in a single cell and its clinical manifestation in a preexisting benign tumor is likely to be long enough to accumulate additional genetic changes in the converted cell. Cell lines have been established from initiated mouse skin or mouse skin papillomas that are phenotypically stable *in vitro* and produce benign tu-

**Table 3. Influence of specific oncogenes on malignant conversion of skin-derived cells.**

| | *In vivo* phenotype | | *In vitro* phenotype |
|---|---|---|---|
| Exogenous gene | Skin graft | Subcutaneous injection | $Ca^{2+}$-induced differentiation |
| Transfection into SP-1 and 308 cells | | | |
| *neo*$^R$ | Papilloma | No growth | Resistant |
| *myc* | Papilloma | No growth | Resistant |
| *EIA* | Papilloma | No growth | Resistant |
| c-*fos* | Papilloma | No growth | Resistant |
| v-*fos* | Carcinoma | Carcinoma | Resistant |
| c-*fos*/v-*fos* chimera | Carcinoma | Carcinoma | Resistant |
| Retroviral infection into normal keratinocytes | | | |
| *neo*$^R$ | Normal skin | No growth | Sensitive |
| v-*fos* | Normal skin | No growth | Sensitive |
| v-Ha-*ras* | Papilloma | Papillomatous cyst | Resistant |
| v-*fos*/v-Ha-*ras* | Carcinoma | Carcinoma | Resistant |

mors when grafted as part of a reconstituted skin in nude mice (*45*). These cells are not tumorigenic by subcutaneous injection. Two cell lines, SP-1 and 308, have been used as recipients for transfected oncogenes to investigate malignant conversion *in vitro* (*46*) (Table 3). Both cell lines contain an activated c-Ha-*ras* gene containing an A to T transversion in codon 61, as expected from their origin in skin exposed to 7,12-dimethylbenz[*a*]anthracene. Plasmid DNAs, encoding a specific oncogene construct, were stably transfected into each cell line. Certain oncogenes were selected because they had been previously shown to cooperate with a *ras* oncogene to enhance transformation in other cell lines. A rearranged murine plasmacytoma-derived c-*myc* gene (minus exon 1; pSVc-*myc*-1) (*42*) or the adenovirus 5 *E1A* gene (1Aneo) (*44*) represented this class. A second oncogene set, chosen because of reported specific expression during embryonic skin development (*47*), included the FBJ v-*fos*(pSVdhfrv-*fos*) (*48*) or a 5′ human c-*fos*/3′FBJ v-*fos*(HVV) (*49*) chimeric transforming construct. All plasmids were used in cotransfections with a neomycin phosphotransferase gene ($neo^R$) contained in $pSV_2neo$ to select for transformants. The products of these oncogenes have a nuclear location and are involved in transcriptional control of other genes. Following transfection and selection in G418 medium, the uptake and expression of exogenous DNA was confirmed in recipient cells by DNA and RNA hybridization analysis (*46*). Transfected cells were then grafted to nude mice or injected subcutaneously, and the tumors were examined histologically and characterized immunohistochemically.

Both *fos* constructs caused malignant conversion in either cell line as defined by the squamous cell carcinoma histology of tumors from grafted cells and the development of carcinomas after subcutaneous injection (*46*). Neither *E1A*, c-*myc*, nor $pSV_2neo$ altered the benign tumor phenotype. Tumors derived by introduction of either of the *fos* oncogenes lacked detectable suprabasal keratin markers using specific antisera and indirect immunofluorescence staining. Tumors from *E1A*, *myc*, or $pSV_2neo$ transfectants expressed low levels of suprabasal keratins in a pattern indistinguishable from parental cells. All tumors expressed basal cell keratins. While a remarkable transformation of the *in vivo* tumor phenotype was induced by transforming *fos* oncogenes, no *in vitro* phenotypic changes were detected. All transfectant lines were similar to the parental cells with regard to cell growth, and all remained anchorage dependent. The expression of the endogenous c-Ha-*ras* mRNA was similar in parental cells and the *fos*-transfected cell lines and tumors, indicating that *fos*-mediated conversion was not through an effect on Ha-*ras* gene expression. The introduction of human proto-oncogene c-*fos* using an endogenous promoter construct did not cause malignant conversion in either cell line, although transcripts for the exogenous gene were detected.

## Analysis of Malignant Conversion by Retroviral Transduction of Genes into Normal Keratinocytes

Since 308 and SP-1 cells could have acquired undocumented genetic changes when established *in vitro*, genes other than the mutated c-Ha-*ras* allele may have contributed to the complementary action of the *fos* oncogene in malignant conversion. To directly test the cooperativity of *ras* and *fos* oncogenes in causing malignant conversion, primary, newborn epidermal cells were used as targets for these genes (*50*) (Table 3). Replication-defective retroviruses were produced by transfecting plasmids containing v-Ha-*ras* or v-*fos* oncogenes into Psi 2 cells, selecting transformant cells, and collecting supernatants (*51*). Newborn mouse keratinocytes were isolated, cultured as basal cells in 0.05 mM $Ca^{2+}$ medium for 2 days, and exposed to retroviral supernatants (*20*). After viral exposure, cells were removed from culture and tested *in vivo* by subcutaneous injection into nude mice or by grafting in a reconstituted skin graft system. A defective retrovirus constructed with $neo^r$ was used as a virus control.

The results of eight independent experiments demonstrated that combined exposure to v-*fos* and v-Ha-*ras* resulted in squamous cell carcinomas by both subcutaneous injection and dermal graft tests within several weeks (*50*). Exposure to only v-Ha-*ras* produced squamous papillomas predominantly. Newborn keratinocytes infected with a v-*fos* retrovirus or a $neo^R$ retrovirus produced normal skin. The benign tumors evolving from v-Ha-*ras* infection expressed K14, K1, and K10 keratins, while the tumors evolving from combined infection with v-*fos* and v-Ha-*ras* expressed K14 but not the suprabasal keratin pair. Nucleic acid hybridization analysis on tumor RNA confirmed that the exogenous oncogenes were expressed appropriately in each group. From these studies it appears that two oncogene changes are sufficient to produce the malignant phenotype in mouse keratinocytes, although the spontaneous occurrence of additional mutational events cannot be ruled out. Interestingly, in the case of the *fos* oncogene, its activation may yield a normal skin phenotype, although cells with this phenotype would be subject to malignant tranformation by activation of a single complementing oncogene.

## Cellular and Molecular Changes Responsible for Malignant Conversion

The induction of the malignant squamous phenotype by cooperation of two oncogenes, v-*fos* and v-Ha-*ras*, provides a good model system to analyze the biochemistry of conversion. It appears that the v-*fos* gene product is the critical element in the conversion event. The c-*fos* protein modulates transcription by forming a heterodimeric complex with AP1, a family of transcription factors that includes the *jun* proto-oncogene (*52,53*). The complex generally increases transcription of other

genes but may downregulate *fos* expression. The v-*fos* transcript is more stable than c-*fos* RNA and encodes an altered protein with an enhanced half-life, thus sustaining the action of *fos* within the nucleus (*54,55*). As a transcriptional enhancer, v-*fos* may act indirectly to induce malignant conversion in keratinocytes by changing the expression of specific cellular genes. Thus, the gene pool regulated by *fos* may be particularly relevant to squamous malignancy.

Experimental analysis has implicated fos/AP1 in regulating a disparate group of genes including α1-collagen, adipocyte P2 (a lipid-binding protein), metallothionein IIA, and two secreted proteases, stromelysin (transin) and collagenase (*55*). Previously, it was shown that stromelysin is commonly elevated in mouse skin carcinomas, but not papillomas (*56*), while collagenase is elevated in many malignancies and has previously been associated with invasion and metastasis (*57*). These secreted enzymes are components of a cascade that includes other classes of proteases. Members of this cascade are secreted as proenzymes, requiring activation themselves by partial proteolysis. A critical activating function is attributed to plasmin, the product of the action of urokinase on the plasmin precursor, plasminogen, which is abundant in circulating fluids (*58*). Our preliminary findings indicate that tumors produced by transfection of v-*fos* plasmids into 308 or SP-1 cells or by infecting primary cells with v-*fos* and v-Ha-*ras*-defective retroviruses have elevated transcripts for stromelysin and urokinase when compared to benign tumors or normal skin (D. A. Greenhalgh et al., manuscript in preparation). Furthermore, analysis of a limited number of chemically induced skin tumors indicates that high levels of both stromelysin and urokinase transcripts are found in carcinomas but not papillomas when compared to normal skin levels.

*In vivo*, malignant conversion is often a variable process that is preceded by progressive dysplastic changes (*59*). Certain oncogenes (such as *neu* and p53) introduced into papilloma cell lines accentuate dysplastic changes in the resultant tumors *in vivo* (*60*) but do not convert to malignancy. The *fos* oncogene may be particularly potent in producing malignant conversion in a single step because it regulates protease secretion, and this could be a pathway that is most direct in producing the malignant phenotype. By invoking a powerful cascade of enzymes in cells already expressing an intrinsic defect in their differentiation program (by virtue of the initiating mutation), the action of the oncogene is amplified. Protease secretion could disrupt protein components of the extracellular environment (e.g., stroma, basement membrane, intercellular matrix, diffusible factors, gradients) that are required to maintain proper structural organization in the benign tumor. Tissue architecture is required for the orderly expression of differentiation markers (*13*). Low levels of protease secretion may be recognized as a dysplastic histotype associated with loss of specific markers when analyzed by molecular probes. As the structural control processes undergo further disruption, severe disturbances in organization, cell-cell relationships, and epithelial-stromal interaction would result in disorderly proliferation and loss of differentiated function.

The proposed involvement of proteases as early effectors in malignant conversion has implications for the mechanism of tumor suppression. The protease cascade is regulated in part by the elaboration of specific inhibitors for individual proteases (*61*). The loss of an inhibitor could result in enhancement of proteolytic activity having similar phenotypic consequences as overexpression of the protease. One implication of such reasoning is that protease inhibitors could compose one class of tumor suppressor genes.

The authors thank Ulrike Lichti for critically reading the manuscript and Margaret Taylor for excellent editorial assistance.

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